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Tubular lipidosis: Epiphenomenon or pathogenetic lesion in human renal disease?

ALBERT C.M. ONG and JOHN F. MOORHEAD

Department of Nephrology and Transplantation, The Royal Free Hospital, London, England, United Kingdom

Tubular lipidosis: Epiphenomenon or pathogenetic lesion in human renal disease? Tubular lipidosis is a commonly observed histological lesion in proteinuric renal diseases. We have studied the interaction between native and modified human lipoproteins and human renal proximal tubular cells to investigate whether lipoproteins could be injurious to tubular cells in culture. Human renal proximal tubular cells were cultured and characterized by established methods. Preliminary studies showed that these cells could take up and degrade normal human lipoproteins by high affinity (HDL) and low affinity (LDL) pathways. In subconfluent culture, native lipoproteins, that is, LDL, HDL_2 and HDL_3, had markedly different effects on cell growth as measured by ^{3}H-thymidine uptake and total cell protein as compared to modified lipoproteins such as minimally modified and oxidized LDL. In addition, we found that renal tubular cells could oxidize native LDL in the presence of copper largely by a superoxide-mediated mechanism. Finally, cellular accumulation of lipid was demonstrated *in vitro* by incubating cultured cells with varying lipoprotein concentrations for up to 48 hours. Notably, cell detachment was observed only with high concentrations of modified LDL especially with minimally modified LDL. We speculate that uptake and oxidation of filtered LDL by tubular cells may lead to tubular injury in nephrotic states.

It is not known how much lipoprotein is normally filtered by the kidney. For instance, HDL-like particles have been detected in concentrated human urine albeit at a higher density range ($d > 1.24$) than plasma HDL ($d = 1.063$ to 1.21) [1]. However, what appears in the urine is probably only a fraction of what is filtered. For example, it has been estimated that up to 3 g of albumin is normally filtered by the kidney in 24 hours but only 30 mg appear in the urine [2].

Several studies have pointed to the fact that unlike normal urine, significant quantities of HDL and lesser amounts of LDL as well as other lipoproteins [3–5] are excreted in nephrotic urine. HDL is the main urinary lipoprotein regardless of serum lipid concentrations, presumably because of its smaller size [4]. In one study, the urinary concentration of Apo-A1, the main apoprotein of HDL, measured using a specific and sensitive immunoassay averaged 38.4 mg/liter in nephrotic patients but was more than a thousandfold less at 16.3 μg/liter in normal controls [6]. The intrarenal handling of filtered lipoproteins is unclear. However, they are probably reabsorbed and catabolized by the proximal tubule as has been shown for HDL_3 in rabbit kidney [7].

There is already a large body of evidence to implicate proteinuria as an independent risk factor in renal disease progression [8]. In pathological states, the major component of urine protein is albumin, but it seems unlikely that albuminuria *per se* is damaging to tubular cells since minimal change disease which is characterized by heavy and sometimes prolonged albuminuria does not lead to progressive tubulointerstitial damage [9]. Moreover, the putative mechanisms by which albuminuria might be damaging are unconvincing [10, 11]. Thus, it is possible that other filtered plasma proteins may account for the pathogenetic role of proteinuria in progression [9]. Furthermore, the known association of acute tubular necrosis with severe nephrotic states, such as with focal segmental glomerular sclerosis (FSGS), has usually been ascribed to renal hypoperfusion but may be due in part to the tubular toxicity of filtered plasma proteins [2]. Significant lipiduria clearly does occur in nephrotic states [12], but the potential toxicity of filtered lipoproteins to renal tubular cells has not been previously examined. Because of species differences for plasma lipoproteins in terms of their normal ratio in plasma [13] and their binding characteristics [14], we chose a human *in vitro* model of tubular lipidosis as the most relevant model for the study of the interaction between filtered lipoproteins and proximal tubular cells.

Methods

Cell culture and characterization

Human renal cortical epithelial cells were grown as previously described from grossly normal renal cortical tissue obtained at nephrectomy or from cadaveric kidneys considered unsuitable for transplantation [15]. The cells were grown on collagen-coated flasks in serum-free hormonally supplemented medium [16] except for the first seven days of culture (5% Nu-serum added). This medium has been found to optimize the growth of human renal tubular cells under serum-free conditions [16].

Tubular cells cultured under these conditions were characterized as a highly enriched population of proximal tubular origin according to previously published methods: ultrastructurally by light and electron microscopy, enzyme histochemistry, immunocytochemistry and sodium-dependent glucose transport [16–18]. Under light microscopy, the cells were of epithelial morphology with a cobblestone appearance. When

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confluent, they formed domes typical of transporting epithelia. By electron microscopy, features typical of proximal tubular epithelia (that is, abundant apical microvilli, mitochondria, endocytic vacuoles and tight junctions) were seen. The cells showed positive surface staining for alkaline phosphatase by the method of Rutenburg and positive intracellular staining for cytokeratin by immunoperoxidase [19]. There was negative staining for factor VIII-related antigen showing the absence of endothelial cells. Sodium-dependent glucose transport was studied using [^{14}C] α-methyl glucopyranoside (AMG), a non-metabolizable analogue of D-glucose, whose uptake by the sodium-glucose cotransporter is specifically inhibited by phlorizin. At one hour, AMG uptake was inhibited to a mean of 52.3% (range 47.7 to 58.2%) in three separate determinations by 0.1 mM phlorizin, similar to the findings of Chung et al [20] for rabbit proximal tubular cells and Kempson et al [17] for human proximal tubular cells.

Preparation of lipoproteins

Human HDL (d = 1.063 to 1.21 g/ml), HDL_2 (d = 1.063 to 1.125 g/ml), HDL_3 (d = 1.125 to 1.21 g/ml) and LDL (d = 1.019 to 1.063 g/ml) were obtained by differential ultracentrifugation flotation of plasma from two normal healthy volunteers as previously described [21]. The isolated fractions were concentrated by further ultracentrifugation and dialyzed extensively against buffer containing 0.15 M phosphate buffered saline (PBS) and 0.1 mM EDTA. LDL used for oxidation was dialyzed against EDTA-free buffer before the addition of copper sulphate (10 μM) as previously described [22]. Minimally modified LDL was generated by prolonged storage of native LDL at 4°C [23]. Thiobarbituric acid reactive substances (TBARS) in the fractions were measured as an indicator of the degree of oxidation [24]. Oxidized LDL was defined as fractions with TBARS of >15 nmol malondialdehyde (MDA) equivalents per mg protein, minimally modified LDL as those with TBARS of 1 to 5 nmol MDA equivalents per mg protein, and non-oxidized LDL as those with TBARS of <0.1 nmol MDA equivalents per mg protein [25]. Lipoprotein fractions were also assessed by changes in electrophoretic mobility on agarose gel (0.7%) electrophoresis [26]. The free radical scavenger, butylated hydroxytoluene (BHT, 20 μM) was subsequently added to both native and modified LDL as an antioxidant. The purity of isolated lipoprotein fractions was assessed by their apoprotein content: HDL and LDL preparations contained less than 1 mg of apo-B and apo-A1 per 100 mg total protein, respectively, as measured by an immunoturbidimetric assay (Technicon RA). All lipoprotein fractions were filtered through a 0.22 μm filter immediately prior to use.

Degradation of lipoproteins

Cellular lipoprotein degradation was determined by measuring the appearance of ^{125}I-iodotyrosine in cell supernatants over time at 37°C as previously described [27]. Freshly isolated lipoproteins (LDL and HDL) were iodinated using lactoperoxidase and glucose oxidase enzymes immobilized on hydrophilic spheres (Enzymobead radioiodination reagent, Biorad Laboratories Ltd, Hemel Hempstead, UK). Na^{125}I was obtained from Amersham International plc (Aylesbury, Bucks, UK). Unbound iodine was removed by passage through a quarternary aminoethyl Sephadex G50 Column (Pharmacia, UK) and exhaustively dialyzed against phophate buffered saline (pH 7.4). Ninety to 99% of the radioactive material was precipitable using 20% trichloroacetic acid (TCA). Radiolabeled lipoprotein preparations were sterilized by passage through a 0.22 μm filter immediately prior to use.

Cells were exposed to incubation medium containing 15 μg/ml of ^{125}I-LDL or ^{125}I-HDL. At predetermined time intervals (15, 30, 45, 60, 120, 180 and 240 min of incubation) dishes were cooled on ice and 0.7 ml of incubation medium with 0.1 ml of pooled human plasma added to tubes containing 2 ml of 14% trichloroacetic acid. Tubes were then centrifuged, incubated on ice for 30 minutes and 2 ml of the supernatant added to 1 ml of 5% $AgNO_3$ solution. After pelleting the silver halide precipitate, aliquots of supernatant were saved to measure ^{125}I radioactivity and the cells dissolved in 0.5 M NaOH for protein estimations [28]. Degradation was expressed as ng ^{125}I-LDL or ^{125}I-HDL degraded per mg cell protein after subtracting any lipoprotein degradation in the absence of cells. Specific activity for HDL was 33.83 cpm/ng protein and for LDL, 46.25 cpm/ng protein.

Oil-red-O staining

Human renal proximal tubular cells were grown to confluency on chamber tissue culture slides (Labtek, Nunc) and incubated with a range of concentrations of human plasma lipoproteins: HDL_3 (100 to 4000 μg/ml), LDL (100 to 4000 μg/ml), minimally oxidized LDL (100 to 3000 μg/ml) and oxidized LDL (100 to 1000 μg/ml) for up to 48 hours. The cells were washed twice with ice-cold PBS and then fixed with 10% neutral-buffered formalin for one hour. Intracellular lipid was stained with oil-red-O and nuclei with hematoxylin. Detached cells were pelleted by centrifugation, resuspended in 0.5 ml of PBS and plated onto a glass slide, air dried and then stained as above.

[^{3}H] thymidine uptake

Cells were plated into 96 well plates at subconfluent density and exposed to "arrest medium" (RPMI 1640 supplemented with antibiotics only) for 48 hours to synchronize the growth phase of all cells to G_o. Lipoprotein containing medium was then added to triplicate or quadruplicate wells at various concentrations for 72 hours; [^{3}H] thymidine (Amersham) was added to each well (1 μCi) 18 hours before the end of the experiment. The cells were washed, trypsinized and harvested onto filter paper using an automatic cell harvester. The amount of [^{3}H] thymidine incorporated into cellular DNA was then estimated by liquid scintillation counting in a beta counter. Results are expressed as percent control to enable comparison between different experiments.

Total cell protein

Cells were plated at subconfluent density (5×10^4 to 2×10^5 cells per 25 cm^2 flask) in basal growth medium. After 48 hours, this was replaced with lipoprotein containing medium at different concentrations in triplicate (day 0). Fresh lipoprotein-containing medium was added at the same concentrations to each flask after 48 hours (day 3). The cells were then solubilized in 0.5 M NaOH for a protein assay at day 5 [28]. To enable comparison between different experiments, total cell protein was calculated as μg/flask and expressed as a percentage of control flasks which received only serum-free medium.

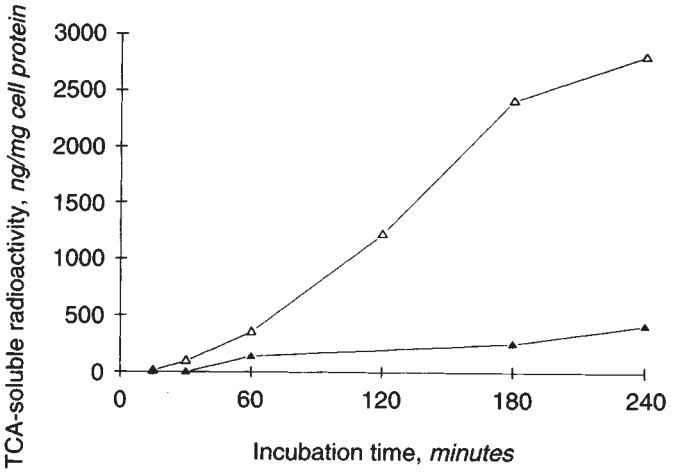

Fig. 1. *Cellular lipoprotein degradation over four hours, measured as trichloroacetic acid (TCA) soluble radioactivity.* Symbols are: (△) LDL; (▲) HDL. Each point represents the mean of four determinations.

Cellular oxidation of LDL

Human renal proximal tubular cells were plated at high density into 12 well plates. After 48 hours, the cells were 80% confluent. Freshly isolated LDL was added (200 μg/ml) in the presence and absence of copper sulphate (5 μM), superoxide dismutase (SOD, 100 μg/ml), 5,8,11,14-eicosatetraynoic acid (ETYA, 20 μM) and nitro-L-arginine methylester (L-NAME, 10 μM) for 24 hours. Preliminary experiments showed that this concentration of copper sulphate does not affect tubular cell viability over a 24 hour period (data not shown) and is consistent with the data of other workers using different cell systems [29]. Identical cell-free wells were set up simultaneously as controls. Oxidation was terminated by the addition of BHT (20 μM) and EDTA (5 μM). The degree of oxidation of LDL was then assessed by TBARS estimation [24].

Materials

Tissue culture medium was obtained from Gibco (Paisley, Scotland, UK) and chemicals from Sigma (Poole, Dorset, UK) unless otherwise stated. Tissue culture flasks and plates were obtained from Falcon (Becton Dickinson, Cowley, UK), benzylpenicillin from Glaxo, bovine dermal collagen (vitrogen 100) from Collagen Corporation (Palo Alto, California, USA), Nuserum from Collaborative Research Inc. (Bedford, Massachusetts, USA), and 0.22 μm biological filters from Millipore (Watford, Herts, UK).

Results

Degradation of lipoproteins

The appearance of ^{125}I iodotyrosine in cell supernatants over time demonstrated that these cells could take up and degrade LDL and HDL (Fig. 1). ^{125}I iodotyrosine could be clearly detected by 30 minutes for LDL and 60 minutes for HDL. There was, however, a clear difference in the magnitude of degradation between LDL and HDL at all time periods. By four hours, lipoprotein degradation had reached 2809 ng/mg cell protein for LDL and 413 ng/mg cell protein for HDL. Counts for "no cell" blanks represented only 0.2% of total added radioactivity.

Oil-red-O (ORO) staining

Both confluent and non-confluent cell layers accumulated intracellular lipid in a dose-dependent manner upon incubation with both native and altered human plasma lipoproteins as shown by positive ORO staining. Of note, no cell detachment was seen with native lipoproteins (HDL_3, LDL) even after prolonged incubation (48 hr) at high concentrations (4000 μg/ml; Fig. 2 a,b). In contrast, oxidative cell damage was seen in subconfluent areas after incubation with oxidized LDL for 24 hours even at low concentrations (100 μg/ml) (data not shown). In confluent cultures, however, oxidative cell damage was not seen but cell detachment (albeit partial) still occurred after a 24 hour incubation with oxidized LDL at high concentration (1000 μg/ml; Fig. 2c). Incubation of both confluent and subconfluent cultures with minimally oxidized LDL resulted in cell rounding (1000 μg/ml) and complete cell detachment (2000 μg/ml) by two hours (Fig. 2d). Detached cells were invariably highly ORO positive with marked intracellular lipid accumulation (Fig. 2d). Interestingly, no cell detachment was observed with cell monolayers incubated with lower concentrations (500 μg/ml or less) of minimally modified LDL in spite of prominent intracytoplasmic lipid accumulation (data not shown). Cells incubated with lipoprotein-free defined medium (Fig. 2e) or delipidated bovine serum albumin (1000 μg/ml) (Fig. 2f) showed no significant ORO positivity.

[^{3}H] thymidine uptake

There was a roughly twofold maximal stimulation at 500 μg/ml lipoprotein followed by a decrease in stimulated thymidine uptake at 5000 μg/ml lipoprotein for both HDL_2 and HDL_3. At all concentrations tested, there was no significant difference in thymidine uptake between HDL_2 and HDL_3 (Fig. 3, Table 1). A decrease in thymidine uptake was observed with both HDL_2 and HDL_3 at concentrations of 5000 μg/ml; this was not significantly different from thymidine uptake by the cells at concentrations of 1000 μg/ml.

A similar pattern of dose-dependent stimulation at low lipoprotein concentrations (10 to 1000 μg/ml) and a non-significant decrease in stimulation at high lipoprotein concentrations (5000 μg/ml) was observed with native LDL (Fig. 4). In contrast, a dose-dependent decrease in thymidine uptake (10 to 1000 μg/ml) associated with cell necrosis and detachment (500 to 1000 μg/ml) was seen following incubation with oxidized LDL (Fig. 4). Cells incubated in parallel for 24 hours with oxidized LDL showed decreased cell viability, as assessed by Trypan blue exclusion: 2% (200 μg/ml) and 0% (500 μg/ml). Compared with LDL, smaller increases in thymidine uptake were seen with minimally modified LDL at all concentrations up to 500 μg/ml, following which thymidine uptake decreased in association with cell detachment (1000 μg/ml; Fig. 5). Cell viability, as assessed by Trypan blue exclusion, also decreased to 33% after a 24 hour incubation with minimally modified LDL at concentrations of 1000 μg/ml but was 90 to 100% at concentrations at or below 500 μg/ml.

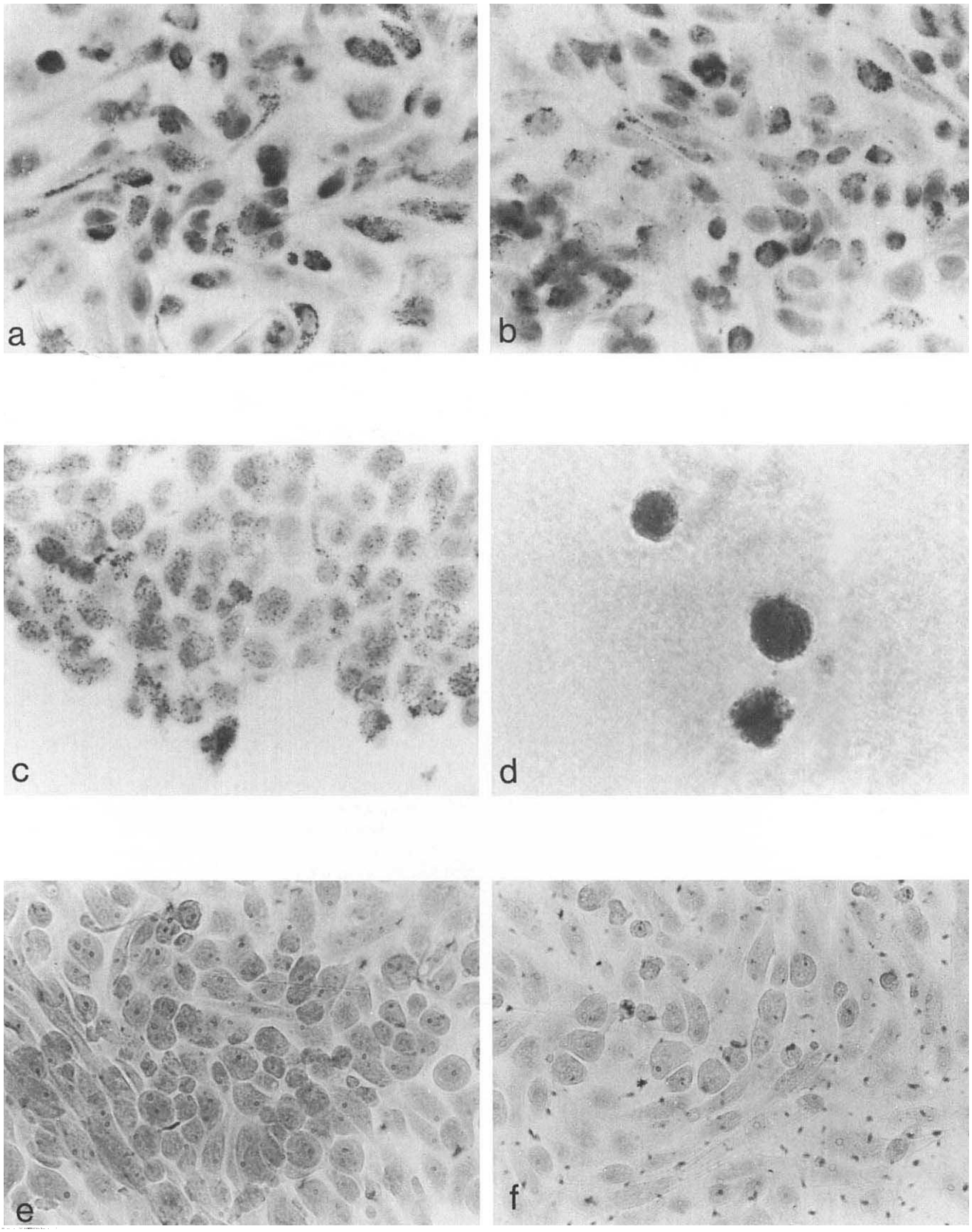

Fig. 2. *Photomicrographs (magnification) of lipid-laden tubular cells following incubation with: (**a**) 4 mg/ml HDL_3 for 48 hours (phase ×400), (**b**) 4 mg/ml native LDL for 48 hours (phase ×400), (**c**) 1 mg/ml oxidized LDL for 24 hours (phase ×400); note the partial cell detachment from a confluent cell monolayer, (**d**) 2 mg/ml minimally modified LDL for 24 hours (phase, oil ×1000); detached cells were air dried onto glass slides and stained with ORO as described in the **Methods** section.* Note the prominent intracytoplasmic inclusions. In contrast, no intracytoplasmic inclusions were seen in cells incubated with (e) defined medium (control) (×400) or (f) delipidated BSA (×400) for 24 hours.

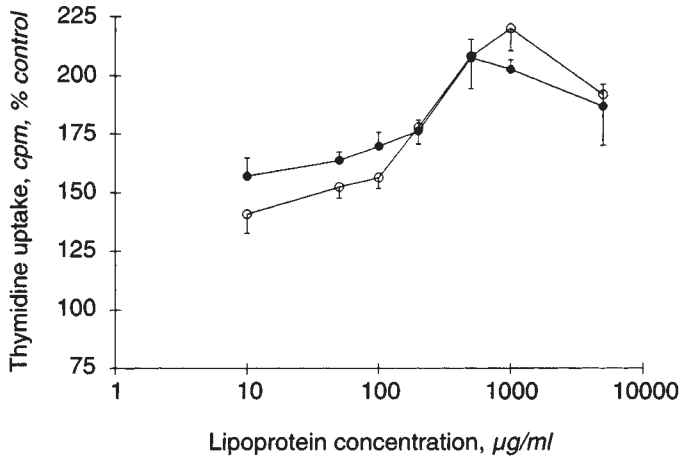

Fig. 3. *^{3}H-thymidine uptake in response to HDL_2 (○) and HDL_3 (●) as compared with lipoprotein-free controls.* Lipoprotein concentration is expressed as μg protein/ml on a logarithmic scale (25 to 5000 μg/ml). Each point represents the mean ± SE of four determinations and is significantly different from control ($P < 0.05$, two-sample *t*-test). There was no significant difference between HDL_2 and HDL_3 at each concentration tested.

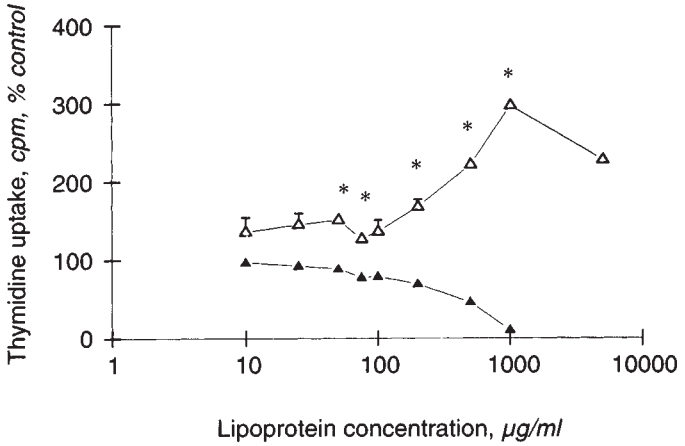

Fig. 4. *Changes in DNA synthesis as measured by ^{3}H-thymidine uptake in response to LDL (△) and oxidized LDL (▲; ox-LDL), in the concentration range 10 to 1000 μg/ml, expressed as a percentage of lipoprotein-free controls (100%).* Each point represents the mean ± SE of four determinations (*$P < 0.05$ wrt ox LDL). For both LDL and ox-LDL, points at or above 200 μg protein/ml were significantly different from control ($P < 0.05$, two-sample *t*-test).

Table 1. [^{3}H]-thymidine incorporation in human PTC after a 72 hours incubation with different concentrations of HDL_2 and HDL_3

Lipoprotein protein concentration μg/ml	[^{3}H]-thymidine incorporation *cpm, % control*	
	HDL_2	HDL_3
10	140.97 ± 8.18	157.10 ± 7.69
50	152.53 ± 4.81	163.94 ± 3.42
100	156.45 ± 4.60	169.84 ± 5.96
200	178.01 ± 7.29	176.28 ± 4.66
500	208.50 ± 14.09	207.65 ± 7.92
1000	220.35 ± 9.58	202.82 ± 3.98
5000	192.06 ± 21.89	186.93 ± 9.44

Total cell protein

Consistent with the data on thymidine uptake, both HDL and LDL stimulated cell growth as measured by total cell protein per flask. There was no significant difference between the maximal growth stimulation produced between both lipoproteins (171.7 ± 10.9% vs. 162.3 ± 5.4%). No difference in cell growth compared with controls was observed over five days at the highest HDL concentration (4.0 mM cholesterol, 8.5 mg/ml protein). This sharp decrease in total cell protein (Table 2) was greater than the maximal decrease in thymidine uptake observed, albeit at lower HDL concentrations (5 mg/ml; Table 1), and may in part reflect the different experimental designs employed.

Cellular oxidation of LDL

In the presence of trace amounts of copper, significant cellular oxidation of LDL was observed, associated with marked oxidative cell damage with membrane blebbing and cell detachment as in the thymidine uptake experiments (Fig. 6b, Table 3). Of the compounds tested, only superoxide dismutase inhibited LDL oxidation appreciably (up to 75%) and although this inhibition was only partial, little histological evidence of

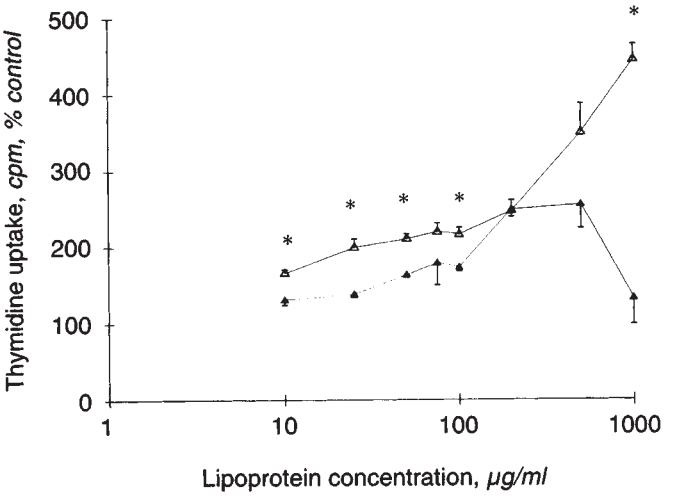

Fig. 5. *Changes in DNA synthesis as measured by thymidine uptake in cells exposed to native LDL (△) or minimally modified (▲; mox-LDL) at concentrations of 10 to 1000 μg/ml, expressed as a percentage of control.* Each point represents the mean ± SE of four determinations (*$P < 0.05$ wrt mox-LDL). Note the smaller increase in thymidine uptake seen with mox-LDL, as compared to LDL, at all concentrations tested.

cell damage was seen in the presence of superoxide dismutase (Fig. 6a). In the absence of copper, superoxide dismutase appeared paradoxically to promote cellular LDL oxidation although the effect was small. Since superoxide dismutase stabilizes nitric oxide (NO), (NO) which is itself a weak oxidant [30] and which has been shown to be produced by tubular cells [31], the effect of co-incubation with L-NAME, an inhibitor of NO synthase was examined in parallel. L-NAME did not abolish this pro-oxidant effect of superoxide dismutase, thus showing that nitric oxide was not responsible. In endothelial cells the 15-lipoxygenase system has been implicated in LDL oxidation [29]. This system is specifically inhibited by ETYA.

Table 2. Effect of HDL and LDL on tubular cell growth measured as changes in total cell protein over five days

Lipoprotein cholesterol concentration (mM)[a,b]	Cell protein per flask *μg/flask, % control*	
	HDL ($N = 6$)	LDL ($N = 3$)
0.25	138.6 ± 3.8	106.6 ± 1.9
0.50	135.1 ± 4.9	115.3 ± 8.5
0.75	171.7 ± 10.9	130.5 ± 4.0
1.00	170.0 ± 2.6	162.3 ± 5.4
2.00	165.5 ± 12.2	155.8 ± 14.5
4.00	107.7 ± 13.6[c]	154.8 ± 10.0

[a] 1 mM HDL cholesterol is equivalent to 1.71 mg/ml HDL protein
[b] 1 mM LDL cholesterol is equivalent to 0.33 mg/ml LDL protein
[c] All values significantly different from control ($P < 0.05$) except HDL cholesterol 4.0 mM ($P > 0.05$)

Our results, however, showed that ETYA had no effect on LDL oxidation by tubular cells.

Discussion

The possibility that filtered lipoproteins are toxic to proximal tubular cells in high concentration (as in heavy proteinuric states) and thus promote progressive tubulointerstitial disease is intriguing but has not been fully explored [32]. Yet as early as 1913, Munk had coined the term "lipoid nephrosis" because he had observed fat bodies in the urine and fatty changes in the tubules of nephrotic patients. He concluded that a "generalized metabolic disorder involving fat metabolism" was the cause [33].

Evidence that tubular uptake of filtered lipoproteins occurs in humans comes from several observations. Nephrotic urine often contains numerous lipid-laden tubular cells [2]. Intracytoplasmic lipid droplets in tubular cells have been demonstrated in renal biopsies from nephrotic patients by electron microscopy [34]. Furthermore, immunohistochemical staining of renal biopsies from nephrotic patients has shown the presence of apoA and apoB, the major apoproteins of HDL and LDL, respectively, in tubular cells and the renal interstitium [35]. It is unlikely though that there is much lipoprotein filtered in minimal change disease, although to date this question has been addressed in only one study [36]. Cellular lipid accumulation in this condition is probably due to the metabolism of albumin-complexed fatty acids to neutral lipids [37]; in this setting, albumin may be considered as a "lipoprotein."

Precise estimates for the amount of filtered lipoprotein in nephrotic states are unavailable, but if it is assumed that like albumin only 1% of the filtered protein load appears in the urine [2], then the average protein concentration of filtered HDL could, for instance, be around 4 mg/ml [6]. This fits in well with a study of two nephrotic patients where calculated estimates of filtered LDL and HDL_2 protein concentrations averaged 4 mg/ml and those of HDL_3 averaged 8 mg/ml [4].

Our first study clearly showed that both HDL and LDL could be taken up and degraded by the cells albeit to different extents. Although lipoprotein binding was not specifically examined, these two patterns of degradation are suggestive of high affinity (LDL) and low affinity (HDL) uptake [38]. Human renal proximal tubular cells appear to possess both receptor and non-receptor mediated mechanisms for LDL uptake [39]. There is evidence that tubular cells possess apical LDL receptors [40, 41] but there is disagreement as to the characteristics and function of these receptors [40–42].

HDL receptors have yet to be fully characterized [43] and their presence or absence on human tubular cells is unknown at present. Nonetheless, our data on HDL degradation would suggest either a non-receptor mediated mechanism or a low-affinity HDL receptor subtype. Luminal HDL_3 uptake can occur in the rabbit straight proximal tubule [7] and this implies a mechanism for reclaiming normally filtered HDL_3. Indeed, if HDL is normally filtered [1], this may be a means of delivering cholesterol to proximal tubular cells [7] in an analogous way as to adrenal and ovary [13], since these cells probably have a higher cholesterol requirement because of their rapid turnover of plasma membrane [44].

However, regardless of the mode of uptake, it is likely that in nephrotic states, proximal tubular cells may become "overloaded" with filtered lipoproteins thus giving rise to the characteristic lipid-laden tubular cells. This profound intracellular lipid accumulation has been assumed to be potentially toxic to the cells [7, 32] but has not been previously examined. Using ORO staining as a marker of lipoprotein uptake, we were able to generate lipid-laden tubular cells *in vitro* by incubating the cells with both normal and modified lipoproteins. Of note, marked cellular lipid accumulation was observed with high concentrations of native HDL_3 and LDL without morphological cell injury (Fig. 2a, b) but incubation with modified LDL, that is, either oxidized (Fig. 2c) or minimally modified LDL (Fig. 2d), resulted in oxidative cell injury and detachment of highly lipid-laden cells. We were unable to examine apical and basolateral lipoprotein uptake separately, as we could not generate sufficiently impermeant cell monolayers (as measured by transmembrane electrical resistance and [^{14}C]-inulin permeability). It is likely though that they are different, as other workers have found in other polarized cells [40, 42].

Our results on the effect of HDL and LDL on cell growth assessed in two different ways show that growth inhibition only occurred at lipoprotein concentrations of 5000 μg/ml or greater. Furthermore, there was no difference between HDL_2 and HDL_3; because of its smaller size, relatively more HDL_3 should be filtered in nephrotic states [45]. These findings are consistent with previous work on MDCK cells for HDL [46]. However, unlike others in other renal cells [21, 46], we did not find native LDL to be toxic to human proximal tubular cells even at high concentrations. It is possible that this reflects the unique capacity of proximal tubular cells to handle a high load of filtered proteins normally [47]. Alternatively, it is possible that small differences in lipid peroxidation which were not measured in earlier studies account for the observed toxicity of native LDL. The mitogenic and cytotoxic effects of LDL on MDCK cells [46] are in fact remarkably similar to our findings with minimally modified LDL. This will be discussed further below.

In contrast, the pattern of growth stimulation and inhibition seen with modified LDL is very different to that seen with normal LDL. Cell detachment was observed with both oxidized and minimally modified LDL at lipoprotein concentrations of 500 μg/ml and 1000 μg/ml, respectively. Brief or extensive oxidation of native LDL is known to lead to the accumulation of

Fig. 7. *E15 rat lung labeled with 2b-11 antisenxe probe.* Grains are most abundant over developing airway epithelium. Much less hybridization is observed over surrounding mesenchyme.
Fig. 8. *E15 rat kidney labeled with 2b-11 anti-sense probe.* (**A**) Grains are most numerous over the comma-shaped bodies (arrows) but are also frequent over ureteric buds (arrowheads). (**B**) Higher power view of S-shaped figure showing intense hybridization over early proximal (P) and distal (D) tubule segments, with somewhat less labeling seen over visceral epithelial cells (arrows).
Fig. 9. *Low* (**A**) *and higher power* (**B**) *micrographs of 3-d-old rat kidney labeled with 2b-11 anti-sense probe.* Note that the most numerous grains overlie early nephric structures in extreme cortex (arrowheads) as well as glomeruli (arrows). Hybridization is also present over collecting ducts (C) but tubular and interstitial areas are generally negative. Reproduction of these figures in color is made possible by a grant from Amersham Life Science, Inc., Arlington Heights, Illinois, USA.

kidney. The reason for the generation of these two different sized transcripts in the kidney is uncertain, but could be related to message stability, which is known to be affected by differential poly-A addition [35]. In addition to differences in transcript size, nucleotide sequence comparisons of the 3′ untranslated regions between the newborn rat kidney clones and mouse and human γ1 cDNAs also showed some deviations. Among these is the insertion of three nucleotides in each of two locations in the rat 3′ untranslated region prior to the first AATAAA polyadenylation signal. Whether any of these differences are functionally important also remains to be established.

The *in situ* hybridization results from fetal rats indicated prominent expression of the laminin γ1 chain mRNA in certain neural structures as well as in the developing gastrointestinal tract, lung, and kidney. These findings are in general agreement with earlier immunofluorescence studies that have localized the γ1 chain to neural, gastrointestinal, lung and kidney basement membranes [31, 32]. In contrast to an earlier report [36], what the present *in situ* hybridization studies show in the lung is that epithelial cells of developing airways express significantly more laminin γ1 chain than the surrounding mesenchyme. In the kidney, there is maximal expression of γ1 mRNA by cells within early glomeruli, although there nevertheless is a persistence of expression during post-natal glomerular and collecting duct growth. In view of recent findings on the loss of specific laminin epitopes from the GBM during glomerular maturation [37], particularly those on the β1 chain [32], the temporal changes seen in glomerular laminin are probably not due to major alterations in synthesis of the γ1 chain. Instead, synthesis of the β2 chain of laminin, which initially appears in the GBM at about the time that β1 declines [32], may signify β chain substitution within the GBM, supporting the concept that laminin isoforms are replaced during GBM maturation [37]. Our *in situ* hybridization results shown here also showed a marked decrease in γ1 message between nascent tubules of forming nephrons and elongating tubules found in the deeper cortex and medulla. These results are also in good agreement with earlier findings showing an abundance of γ1 chain message in RNA obtained from isolated newborn rat glomeruli and an absence in RNA obtained from newborn tubules [20]. Perhaps the truncated γ chain (γ2) recently identified in human medullary collecting duct [14], or some unidentified γ chain isoform substitutes for γ1 during tubule maturation after birth, but this issue needs to be explored further. Alternatively, maybe the high levels of γ1 chain synthesis seen during initial tubulogenesis in embryos provides the full complement of this chain needed for the TBM.

In summary, previous examinations of the expression patterns of laminin in the developing kidney have relied heavily on the premise that the kidney contains laminin corresponding closely to the EHS laminin-1 prototype but numerous other investigations have suggested the presence of other laminin isoforms in the kidney. In the present study, the carboxy terminus of a rat kidney laminin γ chain was cloned and shown to be homologous to the mouse EHS tumor γ1 chain. The region of the laminin molecule represented by the present clones is involved with inter-chain interaction with α1 and β1 laminin polypeptides to form the long arm of the laminin molecule, and this may explain the high degree of homology seen across species in this domain. In addition, the *in situ* hybridization results clearly showed high expression of γ1 chain mRNA during early nephron formation. Although relatively high levels persisted during subsequent glomerular and collecting duct development, γ1 synthesis was downregulated in tubules. The availability of these rat kidney laminin γ1 cDNAs, along with the rat laminin β2 clones [12], should provide improved tools for studying laminin gene expression and protein interactions in the rat kidney.

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Reprint requests to Dale R. Abrahamson, Ph.D., Department of Cell Biology, The University of Alabama at Birmingham, 6th Floor Volker Hall, 1670 University Boulevard, Birmingham, Alabama 35294-0019, USA.

Note Added in Proof

The nucleotide sequence reported in this paper will appear in the EMBL Nucleotide Sequence Database under the accession number X94551.

References

1. Farquhar MG: The glomerular basement membrane. A selective macromolecular filter, in *Cell Biology of Extracellular Matrix* (2nd ed), edited by Hay ED, New York, Plenum Press, 1991, pp 365–418
2. Martin GR, Timpl R, Kuhn K: Basement membrane proteins: Molecular structure and function. *Adv Prot Chem* 39:1–50, 1988
3. Timpl R, Rohde H, Gehron Robey P, Rennard SI, Foidart JM, Martin GR: Laminin – a glycoprotein from basement membranes. *J Biol Chem* 254:9933–9937, 1979
4. Burgeson RE, Chiquet M, Deutzmann R, Ekblom P, Engel J, Kleinman H, Martin GR, Meneguzzi G, Paulsson M, Sanes J, Timpl R, Tryggvason K, Yurchenco PD: A new nomenclature for laminins. *Matrix Biol* 5:209–211, 1994
5. Sasaki M, Kleinman HK, Huber H, Deutzmann R, Yamada Y: Laminin, a multidomain protein. The A chain has a unique globular domain and homology with the basement membrane proteoglycan and the laminin B chains. *J Biol Chem* 263:16536–16544, 1988
6. Sasaki M, Kato S, Kohno K, Martin GR, Yamada Y: Sequence of the cDNA encoding B1 reveals a multidomain protein containing cysteine-rich repeats. *Proc Natl Acad Sci USA* 84:935–939, 1987

Table 3. TBARS generation by tubular cells incubated with native LDL (200 μg) for 24 hours

	TBARS *nmol MDA/mg protein*			
	$-CuSO_4$		$+CuSO_4$ 5 μM	
	Cell	No cell	Cell	No cell
LDL	0	0.41 ± 0.26	70.80 ± 2.49	26.48 ± 2.82
LDL + SOD	4.32 ± 0.52	0	17.36 ± 0.26	3.28 ± 0.26
LDL + ETYA	0	0.41 ± 0.26	70.81 ± 0.52	25.96 ± 0.69
LDL + L-NAME	0	0	71.85 ± 0.78	18.66 ± 0
LDL + SOD + L-NAME	5.63 ± 0.69	2.24 ± 0.78	20.49 ± 0.94	4.84 ± 1.14
LEL + ETYA + L-NAME	0	0.41 ± 0.26	70.54 ± 0.52	19.44 ± 0.45

dismutase. However, the partial nature of this inhibitory effect makes it likely that other oxygen free radicals such as hydroxyl radicals and hydrogen peroxide [55] are also involved. The latter is a weak oxidant of LDL but its formation is enhanced by superoxide dismutase [56]; this may account for the small rise in TBARS noted when LDL was incubated with superoxide dismutase alone (Table 2).

Clearly, whether or not lipoproteins are oxidized *in vivo* in the diseased kidney will depend on the balance between antioxidants and pro-oxidants present [57]. In our system as in previous work, to demonstrate oxidation of LDL by tubular cells we excluded serum as well as other antioxidants (apart from transferrin) and added trace amounts of copper [48, 52, 53]. Since a variety of plasma antioxidants and intrinsic renal antioxidant enzymes are normally present *in vivo* [57], the minimal modification of LDL may more closely reflect events occurring in the diseased kidney. Moreover, the marked oxidative damage which we observed in cells incubated with LDL and copper (Fig. 6b) is not usually observed *in vivo*. In this context, it is particularly striking that high concentrations of minimally modified LDL (1 mg/ml) were toxic to confluent monolayers of human proximal tubular cells and complete cell detachment from a confluent monolayer seen following incubation with 2 mg/ml minimally modified LDL (Fig. 2d). Lipoprotein uptake appeared to be rapid as the cells were detaching from their culture surface within 90 minutes, and appeared to be full of intracellular lipid as demonstrated with oil-red-O staining (Fig. 2d). In contrast, oxidized LDL caused incomplete cell detachment from a confluent monolayer at 24 hours even though the cells that remained attached contained numerous lipid inclusions (Fig. 2c). It seems likely that this difference is related to a difference in the mode of uptake of each lipoprotein. Oxidized LDL is probably taken up by endocytosis and it may itself inhibit endocytosis by confluent cells [48]. This may in turn partly limit its toxicity on confluent cell monolayers in contrast to its effects on subconfluent cells [49]. On the other hand, minimally modified LDL is recognized by the classical LDL receptor and is taken up rapidly. Thus, potentially toxic lipid oxidation products such as lipid peroxides and cholesterol epoxides [25] may accumulate rapidly at high concentrations intracellularly and cause cell detachment. This appears to be a concentration-dependent phenomenon since lower concentrations of minimally modified LDL did not lead to cell detachment.

In summary, we have demonstrated that modified lipoproteins are toxic to human renal proximal tubular cells in culture. Under certain conditions, human renal proximal tubular cells may also oxidize LDL. In contrast, marked intracellular lipid accumulation can be generated in tubular cells using high concentrations of native lipoproteins without causing apparent cell injury. Thus in nephrosis, one possible scheme by which filtered lipoproteins might be toxic to proximal tubular cells is as follows: filtered lipoproteins are taken up by proximal tubular cells thus generating lipid-laden cells. Depending on the balance of oxygen radicals generated and anti-oxidants present, tubular cells may modify LDL. Increased oxygen free radical production could be a consequence of increased metabolic work in remnant nephrons (which would also be filtering and handling lipoprotein) [58] or because of hypoxia [59]. Hematuria [60] and transferrinuria [61], being common accompaniments of proteinuria, could provide a ready source of iron to catalyze the oxidative process. Alternatively, LDL may be oxidized prior to filtration by resident or inflammatory glomerular cells. The toxic effects of oxidized LDL could thus lead to oxidative tubular injury and the shedding of lipid-laden tubular cells in nephrotic urine.

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Reprint requests to Dr. A.C.M. Ong, Department of Medicine, UCL Medical School, The Rayne Institute, University St., London WC1E 6JJ, England, United Kingdom.

References

1. Segal P, Gidez LI, Vega GL, Edelstein D, Eder HA, Roheim PS: Apoproteins of high density lipoproteins in the urine of normal subjects. *J Lipid Res* 20:784–788, 1979
2. Olson JL: The nephrotic syndrome, in *Pathology of the Kidney*, edited by Heptinstall RH, Little, Brown & Co, Boston, 1992, pp 779–870
3. Saku K, Reddy GS, Hynd BA, Kashyap ML: Renal handling of high density lipoproteins by isolated perfused kidneys. *Metabolism* 33:432–438, 1984
4. Shore VG, Forte T, Licht H, Lewis SB: Serum and urinary lipoproteins in the human nephrotic syndrome: Evidence for renal catabolism of lipoproteins. *Metabolism* 31:258–268, 1982
5. Moorhead JF, El-Nahas M, Harry D, Persaud JW, Mayne K, Chan MK, Varghese Z: Focal glomerular sclerosis and nephrotic syndrome with partial lecithin:cholesterol acyltransferase deficiency and discoidal high density lipoprotein in plasma and urine. *Lancet* 1:936–937, 1983

6. Gomo Zar, Henderson LO: High density lipoprotein apoproteins in urine: II. Enzyme-linked immunoassay of apolipoprotein A-1. *Clin Chem* 34:1781–1786, 1988
7. Peterson DR, Hjelle JT, Carone FA, Moore PA: Renal handling of plasma high density lipoprotein. *Kidney Int* 26:411–421, 1984
8. Remuzzi G, Bertani T: Is glomerulosclerosis a consequence of altered glomerular permeability to macromolecules? *Kidney Int* 38:384–394, 1990
9. Ong ACM, Fine LG: Loss of glomerular function and tubulointerstitial fibrosis: Cause or effect? *Kidney Int* 45(Suppl 45):1994
10. Eddy AA: Interstitial nephritis induced by protein-overload proteinuria. *Am J Pathol* 135:719–733, 1989
11. Olbricht CJ, Cannon JK, Garg JC, Tisher CC: Activities of cathepsins B and L in isolated nephron segments from proteinuria and non-proteinuric rats. *Am J Physiol* 250:F1055–F1062, 1986
12. Jungst D, Caselmann WH, Kutschera P, Weisweiler P: Relation of hyperlipidemia in serum and loss of high density lipoproteins in urine in the nephrotic syndrome. *Clin Chim Acta* 168:159–167, 1987
13. Brown MS, Kovanen PT, Goldstein JL: Receptor-mediated uptake of lipoprotein-cholesterol and its utilization for steroid synthesis in the adrenal cortex. *Rec Prog Hormon Res* 35:215–249, 1979
14. Kramer A, Pavenstadt H, Nauck M, Wieland H, Schollmeyer P, Wanner C: LDL uptake and cholesterol metabolism in mesangial cells. (abstract) *Kidney Int* 44:248–249, 1993
15. Ong ACM, Jowett TP, Scoble JE, O'Shea JA, Varghese Z, Moorhead JF: The effect of cyclosporin A on endothelin synthesis by cultured human renal cortical epithelial cells. *Nephrol Dial Transplant* 8:748–753, 1993
16. Detrisac CJ, Sens MA, Garrin AJ, Spicer SS, Sens DA: Tissue culture of human kidney epithelial cells of proximal tubule origin. *Kidney Int* 25:383–390, 1984
17. Kempson SA, Mcateer JA, Al-Mahrouq HA, Dousa TP, Dougherty GS, Evan AP: Proximal tubule characteristics of cultured human renal cortex epithelium. *J Lab Clin Med* 113: 285–296, 1989
18. Schmouder RL, Strieter RM, Wiggins RC, Chensue SW, Kunkel SL: *In vitro* and *in vivo* interleukin-8 production in human renal cortical epithelia. *Kidney Int* 41:191–198, 1992
19. Dacie JV, Lewis SM: Blood cell cytochemistry and supplementary techniques, in *Practical Haematology*, Edinburgh, Churchill Livingstone, 1984, p 87
20. Chung SD, Alavi N, Livingston D, Hiller S, Taub M: Characterization of primary rabbit kidney cultures that express proximal tubule functions in a hormonally defined medium. *J Cell Biol* 95:118–126, 1982
21. Wheeler DC, Persaud JW, Fernando R, Sweny P, Varghese Z, Moorhead JF: Effects of low density lipoproteins on mesangial cell growth and viability *in vitro*. *Nephrol Dial Transplant* 5:185–191, 1990
22. Heinecke JW, Baker L, Rosen H, Chait A: Superoxide-mediated modification of low density lipoprotein by arterial smooth muscle cells. *J Clin Invest* 77:757–761, 1986
23. Rajavashisth TB, Andalibi A, Territo MC, Berliner JA, Navab M, Fogelman AM, Lusis AJ: Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. *Nature* 344:254–257, 1990
24. Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL, Steinberg D: Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc Natl Acad Sci USA* 81:3883–3887, 1984
25. Berliner JA, Territo MC, Seranian A, Ramin S, Kim JA, Bamshad B, Esterson M, Fogelman AM: Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *J Clin Invest* 85:1260–1266, 1990
26. Noble RP: Electrophoretic separation of plasma lipoproteins in agarose gel. *J Lipid Res* 9:357–364, 1968
27. Wheeler DC, Fernando RL, Gillett MPT, Zaruba J, Persaud J, Kingstone D, Varghese Z, Moorhead JF: Characterization of the binding of low density lipoproteins to cultured rat mesangial cells. *Nephrol Dial Transplant* 6:701–708, 1991
28. Markwell MAK, Haas SM, Bieber LL, Tolbert NE: A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* 87:206–210, 1978
29. Parthasarathy S, Wieland E, Steinberg D: A role for endothelial cell lipoxygenase in the oxidative modification of low density lipoprotein. *Proc Natl Acad Sci USA* 86:1046–1050, 1989
30. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA: Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 87:1620–1624, 1990
31. Ishii K, Chang B, Kerwin JF, Wagenaar FL, Huang Z-H, Murad F: Formation of endothelium-derived relaxing factor in porcine kidney epithelial LLC-PK_1 cells: An intra- and intercellular messenger for activation of soluble guanylate cyclase. *J Pharmacol Exp Ther* 256:38–43, 1991
32. Moorhead JF, Chan MK, El-Nahas M, Varghese Z: Lipid nephrotoxicity in chronic progressive glomerular and tubulointerstitial disease. *Lancet* 2:1309–1311, 1982
33. Munk F: Klinische diagnostik der degenerativen nierenerkrankungen. *Z Klin Med* 78:1, 1913
34. Neverov N, Ivanov A, Severgina E, Kolonduck N, Srinivas K, Tareyeva I: Cytoplasmic lipid inclusions and low-density lipoprotein depositions in renal biopsies of nephrotic patients. (abstract) *Nephrol Dial Transplant* 10:776, 1991
35. Kashyap ML, Ooi BS, Hynd RA, Glueck CJ, Pollak VE, Robinson K: Sequestration and excretion of high density and low density lipoproteins by the kidney in human nephrotic syndrome. *Artery* 6:108–121, 1979
36. Lopes-Virella M, Virella G, Debeukelaer M, Owens CJ, Colwell JA: Urinary high density lipoprotein in minimal change glomerular disease and chronic glomerulopathies. *Clin Chim Acta* 94:73–81, 1979
37. Thomas M, Schreiner GF: Proximal tubule (PT) cells show differential metabolism of endocytosed fatty acids (FA) into neutral lipids. (abstract) *J Am Soc Nephrol* 3:752, 1992
38. Goldstein JL, Brown MS: Binding and degradation of low density lipoproteins by cultured human fibroblasts. *J Biol Chem* 249:5153–5162, 1974
39. Chatterjee S, Clarke KS, Kwiterovich PO: Uptake and metabolism of lactosylceramide on low density lipoproteins in cultured proximal tubular cells from normal and familial hypercholesterolemic homozygotes. *J Biol Chem* 261:13480–13486, 1986
40. Li C, Stifani S, Schneider WJ, Poznansky MJ: Low density lipoprotein receptors on epithelial cell (Madin-Darby Canine Kidney) monolayers. *J Biol Chem* 266:9263–9270, 1991
41. Pathak RK, Yokode M, Hammer RE, Hofmann SL, Brown MS, Goldstein JL, Anderson RGW: Tissue-specific sorting of the human LDL receptor in polarized epithelia of transgenic mice. *J Cell Biol* 111:347–359, 1990
42. Hashimoto S, Nagami GT: Low density lipoprotein uptake and cholesterol accumulation by cultured renal cells. *J Am Soc Nephrol* 2:1101–1107, 1991
43. Graham DL, Oram JF: Identification and characterization of a high density lipoprotein-binding protein in cell membranes by ligand blotting. *J Biol Chem* 262:7439–7442, 1987
44. Bode F, Baumann K, Kinne R: Analysis of the pinocytic process in rat kidney. II. Biochemical brush border microvilli, lysosomes and basolateral plasma membranes. *Biochim Biophys Acta* 433: 294–310, 1976
45. Scanu AM: Physiopathology of plasma lipoprotein metabolism. *Kidney Int* 39(Suppl 31):S3–S7, 1991
46. Gospodarowicz D, Cohen DC, Massoglia SL: Stimulation of the proliferation of the Madin-Darby Canine Kidney (MDCK) epithelial cell line by high-density lipoproteins and their induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. *J Cell Physiol* 117:76–90, 1983
47. Carone FA, Peterson DR, Oparil S, Pullman TN: Renal tubular transport and catabolism of proteins and peptides. *Kidney Int* 16:271–278, 1979
48. Steinbrecher UP, Zhang H, Lougheed M: Role of oxidatively

modified LDL in atherosclerosis. *Free Radical Biol Med* 9:155–168, 1990
49. KOSUGI K, MOREL DW, DICORLETO PE, CHISHOLM GM: Toxicity of oxidized low density lipoprotein to cultured fibroblasts is selective for the S-phase of the cell cycle. *J Cell Physiol* 102:119–127, 1987
50. WITZTUM JL, STEINBERG D: Role of oxidized low density lipoprotein in atherogenesis. *J Clin Invest* 88:1785–1792, 1991
51. ALOMAR Y, NEGRE-SALVAYRE A, LEVADE T, VALDIGUIE P, SALVAYRE R: Oxidised HDL are much less cytotoxic to lymphoblastoid cells than oxidised LDL. *Biochim Biophys Acta* 1128:163–166, 1992
52. PARTHASARATHY S, PRINTZ DJ, BOYD D, JOY L, STEINBERG D: Macrophage oxidation of low density lipoprotein generates a modified form recognised by the scavenger receptor. *Arteriosclerosis* 3:149–159, 1983
53. MOREL DW, DICORLETO PE, CHISHOLM G: Endothelial and smooth muscle cells alter low density lipoproteins in vitro by free radical oxidation. *Arteriosclerosis* 4:357–364, 1984
54. FERNANDO RL, VARGHESE Z, MOORHEAD JF: Oxidation of low-density lipoproteins by rat mesangial cells and the interaction of oxidized low-density lipoproteins with rat mesangial cells *in vitro*. *Nephrol Dial Transplant* 8:512–518, 1993
55. ROVIN BD, WURST E, KOHAN DE: Production of reactive oxygen species by tubular epithelial cells in culture. *Kidney Int* 37:1509–1514, 1990
56. CATHCART MK, MCNALLY AK, CHISOLM GM: Lipoxygenase-mediated transformation of human low density lipoprotein to an oxidised and cytotoxic complex. *J Lipid Res* 32:63–70, 1991
57. YOSHIOKA T, BILLS T, MOORE-JARRETT T, GREENE HL, BURR IM, ICHIKAWA I: Role of intrinsic antioxidant enzymes in renal oxidant injury. *Kidney Int* 38:282–288, 1990
58. NATH KA, CROATT AJ, HOSTETTER TH: Oxygen consumption and oxidant stress in surviving nephrons. *Am J Physiol* 258:F1354–F1362, 1990
59. PALLER MS, NEUMANN TV: Reactive oxygen species and rat renal epithelial cells during hypoxia and reoxygenation. *Kidney Int* 40:1041–1049, 1991
60. HILL PA, DAVIES DJ, KINCAID-SMITH P, RYAN GB: Ultrastructural changes in renal tubules associated with glomerular bleeding. *Kidney Int* 36:992–997, 1989
61. ALFREY AC, FOMENT DH, HAMMOND WS: Role of iron in tubulointerstitial injury in nephrotoxic serum nephritis. *Kidney Int* 36: 753–759, 1989